Plant Molecular Biology 3: 45-58 (1984).
© Martinus Nijhoff/Dr W. Junk Publishers, The Hague, Printed in the Netherlands.

Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs

Eva Czarnecka, L. Edelman, F. Schöffl & Joe L. Key*
Botany Department, University of Georgia, Athens, GA 30602, U.S.A.

Keywords: heat shock, physical stresses, cDNA clones, northern blot hybridization analysis

Summary

Soybean seedlings were subjected to a wide range of physical (abiotic) or environmental stresses. Cloned cDNAs to heat shock (hs)-induced mRNAs were used to assess whether these diverse stresses induced the accumulation of poly(A)RNAs in common with those induced by hs. Northern blot hybridization analyses indicated that a wide range of stress agents lead to the accumulation of detectable levels of several of the hs-induced poly(A)RNAs; the relative concentration of those RNAs 'induced' by the wide range of stress agents (e.g. water stress, salt stress, anaerobiosis, high concentrations of hormones, etc.), was generally in the order of 100-fold lower than that induced by hs. There are two notable exceptions to that pattern of response to the stress agents. First, arsenite treatment resulted in accumulation of the 'hs poly(A)RNAs' to levels similar to those induced by hs. Cadmium also induced a somewhat normal spectrum of the 'hs poly(A)RNAs', but generally lower levels accumulated than in hs- and arsenite-treated tissues. Second, one set of poly(A)-RNAs which are present at low and variable levels in control (non-stressed tissue) tissue, and which are increased some 5- to 10-fold by hs, increased in relative concentration in response to a wide range of the stress agents similarly to the response to hs. The physiological significance of the accumulation of this set of poly(A)-RNAs (which translate into four electrophoretically different 27 kd proteins) is not known, but they certainly seem to serve as a monitor (or barometer) of physiological stress conditions. Cadmium treatment results in the accumulation of those same poly(A)RNAs and an additional band of higher molecular weight poly(A)-RNA homologous to the same hs cDNA clone (clone pCE 54). Ethylene seems to have no obvious causal relationship to the hs response, even though hs-treated seedlings display some symptoms similar to those exhibited by ethylene-treated seedlings.

Introduction

The protein synthetic system of plants responds rapidly to the imposition of stress including anaerobiosis and DNP treatment (34), lowering of the water potential (24, 43), and heat shock (28). Polyribosomes rapidly deplete to very low levels with the completion of read-out of nascent proteins and their release followed by the accumulation of low levels of polyribosomes (e.g. 28, 34). There is preservation of the normal mRNAs during the stress treatment (34, 51, 54), and this mRNA can be recruited into active protein synthesis following relief from the stress condition. In addition there is a marked change in protein synthesis during the stress treatment (4, 8, 28, 51); generally the synthesis of most normal proteins is reduced, and synthesis of a new set of proteins is induced. A unique set of anaerobic proteins is induced in corn seedlings during anaerobiosis, including alcohol dehydrogenase (14, 51). Rice seedlings seem to show a similar response to anoxia as does corn (41). Heat shock (hs) of a wide range of plant systems studied to date results in the appearance of a new set of proteins (hs

* To whom all reprint requests should be addressed.

proteins) and depressed synthesis of most normal proteins (4, 5, 12, 28, 53). The appearance of alcohol dehydrogenase during anaerobiosis and the hs proteins during heat shock results from the production and accumulation during the stress treatment of mRNAs for those proteins (18, 54).

While the response to heat shock seems to be ubiquitous, occurring in a diverse range of organisms from bacteria to man (3, 15, 20, 27, 35, 37, 44, 61) including plants as noted above, the *Drosophila* system is the most studied of those (3). That the heat shock response of *Drosophila* relates in part to altered patterns of transcription came from studying puffing and polythene chromosomes in response to elevated temperatures (50) and has since been verified by a number of studies using molecular approaches. The response also includes translation controls, since hs mRNAs are selectively translated relative to the normal mRNAs which persist during hs (55, 57).

In addition to heat shock, the *Drosophila* system responds to a wide range of stress agents (e.g. DNP, arsenite, release from anoxia) by altered puffing patterns (13) and synthesis of the hs proteins (3). In *Tetrahymena*, deciliation, release from anoxia and hs result in the synthesis of a common set of proteins (20). Amino acid analogues induce a set of proteins in chick fibroblasts similar to those induced by hs (27). Arsenite seems to induce a set of proteins similar to hs proteins in a number of systems (3, 26, 32, 60). These observations have led to the view that organisms may respond to a wide range of stress agents at the level of transcription and translation to produce a set of proteins which provide some kind of physiological protection or homeostatic state (3). Certainly, the accumulation of hs proteins during heat shock seems to provide thermal protection to otherwise non-permissive temperatures in a wide range of organisms (2, 29, 33, 36, 37, 40, 61).

All of these observations taken together prompted us to initiate an investigation of the response of plant tissues to a wide range of stress agents. We wished to ascertain if some common regulatory mechanism might respond to diverse stress signals in plants resulting in the induction of synthesis of a common set of mRNAs and proteins. As one approach to this analysis, cDNA clones to hs mRNAs of soybean (54) were used to assess whether a wide range of environmental (abiotic) stress agents induce a set of mRNAs common to those induced by hs. This approach was used as a primary evaluation instead of analysis of *in vivo* synthesized proteins in response to the stress agents because of the difficulty of achieving reasonable levels of amino acid uptake and incorporation into protein during most of the stress treatments (e.g. DNP, water stress, etc.). Also the hybridization of poly(A)RNAs to the cloned hs cDNAs allows direct identification of an mRNA induced in response to some stress agent with a hs mRNA. Our results show that a wide range of stress agents induce the accumulation of some hs mRNAs similarly to hs, while some hs mRNAs are not induced at all or only at very low levels relative to hs by many of the stress agents tested. Of those stress agents studied in any detail, arsenite seems to more precisely mimic hs than the others (this also has been verified by *in vivo* protein synthesis during arsenite treatment; unpublished data).

Materials and methods

Plant material; incubation conditions

Soybean seedlings (*Glycine max* variety Wayne) were grown in moist chem-pak bags at 28 °C in the dark for two days (length of seedlings was 2–3 cm). The cotyledons were removed, and the seedlings were used for further treatments. Alternatively, seeds were germinated in moist vermiculite for four days (at 28 °C in the dark) with 1 cm slices of mature hypocotyl being used for incubation. The tissue incubations were carried out in shaking water baths in 250 ml erlenmeyer flasks containing Solution A: 1% sucrose, 1 mM KPO_4 buffer pH 6.0, 50 μg/ml chloramphenicol and 10 μg/ml 2,4-dichlorophenoxyacetic acid, the latter only in case of mature hypocotyl tissue. Different stress treatments are indicated in the figure legends. In order to achieve anaerobic conditions, N_2 gas was gently bubbled into the incubation medium. Ethylene treatment (10 parts per million in air) was achieved as with anaerobiosis. Low water potential was created by using different concentrations of polyethylene glycol (PEG-6000). At 28° C the following PEG-6000 concentrations gave the desired osmotic potentials: 124 g/Kg H_2O, -2 bars; 208 g/Kg H_2O, -5 bars; 269 g/Kg H_2O, -8 bars; 319 g/Kg H_2O, -11 bars; 363 g/Kg H_2O, -14 bars (39).

Purification of poly(A)⁺RNA

Total RNA was extracted from soybean seedlings or excised hypocotyl tissues after 2 h incubation (unless stated differently) under control (28 °C in solution A) or stress conditions, according to Silflow *et al.* (56), with the modification of replacing the CsCl step by 3.0 M NaCl precipitation (6). Alternatively, total RNA was extracted by the TNS/PAS method (25) where homogenization of the tissue was accomplished in the detergent buffer prior to the addition of phenol. Polyadenylated RNA was obtained from total RNA by oligo(dT) cellulose chromatography (56), and quantitated by [^{3}H]-poly(U) hybridization (9).

Northern blot hybridization analysis of RNA

Polyadenylated RNA samples (1 μg) were electrophoresed on 2% agarose, 6% formaldehyde gels (47) and transferred overnight to nitrocellulose filters (59). Filters were dried, baked at 80 °C under vacuum for 2–3 h, subsequently prehybridized at 42 °C for 3 h and hybridized overnight to selected nick-translated cDNA clones according to Baulcombe & Key (6). The hybridization mixture was then removed and stored at -20 °C for further use. The filters were washed three times in 2 × SSC, 0.1% SDS for 5–10 min each and three times in 0.1 × SSC, 0.1% SDS at room temperature. Dried filters were autoradiographed, usually overnight, at -70 °C with cronex lighting plus intensifying screens and Kodak XR films as described by Swanstrom & Shank (58).

Dot blot hybridization analysis of RNA

Dot blots were performed as described by Thomas (59) using a hybri-dot manifold from BRL. Nitrocellulose filters were first equilibrated with H_2O, then 20 × SSC and placed in hybri-dot manifold after removing the excess 20 × SSC. Samples of polyadenylated RNA (amounts from 0.001–0.1 μg in 50 μl volume of 10 to 20 × SSC) were applied in the wells of the manifold under slight vacuum. Subsequently, each sample well was washed with several volumes of 20 × SSC. Filters were dried at room temperature, baked in a vacuum oven at 80 °C for 2 h, prehybridized, hybridized, washed and autoradiographed as for Northern blot filters (described above).

Nick translation of the plasmids

Soybean heat shock-specific cDNA clones selected by *in situ* colony hybridization were purified and labeled with [α-^{32}P] dCTP from Amersham (s.a. 400 Ci/m mol) by nick translation (48) to a specific activity of 4×10^7 – 10×10^7 cpm/μg DNA. The unincorporated nucleotide was separated from labeled cDNA by chromatography on Sephadex G-50 or by the Spun-column procedure (42).

Selection of stress-specific recombinant cDNA clones

cDNA recombinant clones were constructed using polyadenylated RNA from heat shock hypocotyl (54). The recombinant cDNA library was screened as described by Schöffl & Key (54). The set of transformants was grown on selective nutrient agar plates, replicated and immobilized on several filters for further differential replicate hybridizations. Each set of filters was hybridized to labeled cDNA [α-^{32}P] dCTP, s.a. 400 Ci/m mol, Amersham) synthesized from various polyadenylated RNA templates isolated from soybean seedlings treated by different environmental stresses. Clones were selected which showed significant increase of the signal on autoradiographs when screened with cDNAs from several stress conditions over the signal with the control cDNA. Three such clones were identified and used in subsequent analyses along with those isolated previously (54).

cDNA synthesis

cDNA synthesis from polyadenylated RNA was performed according to Silflow *et al.* (56) using RNAs from tissue incubated under various stress conditions (e.g. low water potential, anaerobiosis, high salt concentration, 2,4-dinitrophenol). As a control, the RNA from untreated soybean seedlings was used. The reverse transcriptase used for cDNA synthesis was received from Dr. J. Beard of the National Cancer Institute.

Plasmid preparation

Plasmid DNA was obtained using the saracosyl method (7). The procedure involved an overnight chloramphenicol treatment of the cells before extraction (21).

Hybrid release translation

cDNA clones were immobilized to nitrocellulose filters (BA85) according to Raskas & Green (46). Hybridization of plasmid to complementary poly(A)$^+$RNA was performed as described by McGrogan *et al.* (38). After several washes removing nonspecifically bound RNA, specifically bound mRNA was eluted from the filter by thermal wash at 85 °C with a solution containing 0.002 M EDTA and 10 μg/ml wheat germ tRNA. RNA to be used for *in vitro* translation was ethanol precipitated from the eluate made 0.1 M KOAc and 0.01 M $MgCl_2$.

In vitro translation of hybrid selected mRNA was carried out in the cell free wheat germ S-30 system containing placental RNAse inhibitor as described by Key *et al.* (28). About 0.05 μg of selected poly(A)$^+$RNA was translated in the assay.

One dimensional SDS gel electrophoresis of the translation products was done according to Laemmli (31) and two dimensional gel electrophoresis as described by O'Farrell (45).

Results

Isolation of cDNA recombinant clones homologous to stress-specific polyadenylated RNA

The cDNA recombinant clone library constructed from hs-induced poly(A)RNA of soybean (54) was differentially screened by *in situ* colony hybridization with [^{32}P] cDNA probes prepared from control (28 °C) RNA and RNA isolated from soybean seedlings which were heat shocked (40 °C) or treated with 2,4-dichlorophenoxyacetic acid (510 μg/ml), low water potential (-6 bars of PEG 6000), abscisic acid (0.4 mM), N_2 gas and KCl (250 mM). Out of 500 transformants screened, four colonies were selected which hybridized with cDNAs made from all samples of 'stress' RNA (data not shown). Two of these hybridized weakly to control cDNA, but equally strong signals were obtained with the different 'stress' cDNAs. These two clones were subsequently shown to represent the same (or highly homologous) sequence, and will be referred to subsequently as clone pCE 54. Two additional colonies were selected which gave strong signals with hs-cDNA, no signal with 28 °C cDNA, and varying signals with the 'stress' cDNAs (clones pCE 53 and pCE 75). Clones pCE 53 and pCE 75 hybridized to RNA of about 950 bases in length on northern blots while clone pCE 54 hybridized to a slightly larger band of RNA. The insert size of clone 54 was 540 base pairs, that of clone 53 about 325 base pairs and clone 75 approached full length. These three clones, along with the group isolated earlier (pFS 2005, 2019, 2033) (54) were used in subsequent northern blot hybridization and hybrid selection analyses of the various 'stress' RNAs.

Induction of stress-specific poly(A)RNA

The influence of a number of different stresses on RNAs homologous to the five different hs cDNA clones is presented in Fig. 1. A wide range of stress agents induces low levels of RNAs homologous to the group of clones reported in earlier hs studies (54); yet the level of accumulation of these mRNAs in response to this group of stresses is very small relative to that induced by hs as noted in lanes 13 and 15 (Fig. 1A). Arsenite induces significant levels of RNA homologous to clone 2005, but somewhat lower than hs (Fig. 1B, lanes 2 and 6); much lower levels of this RNA accumulate in tissue treated with DNP and canavanine, with only trace or undetectable levels occurring in other treatments. A similar pattern is seen for clone 53 (Fig. 1C) as for clone 2005, with arsenite inducing levels approaching that of hs (lanes 2 and 6) with lower to undetectable levels accumulating in response to the other treatments. Arsenite and hs cause the accumulation of similar levels of poly(A)RNA hybridizing to clone 75 (Fig. 1D) with only trace or undetectable levels occurring in response to the other stresses. All stresses analyzed in this experiment caused the enhanced accumulation of poly(A)RNA hybridizing to clone 54 (Fig. 1E).

Several metals which are known to be toxic to plants at high levels were tested for their influence on the accumulation of 'hs-specific' mRNAs (Fig. 2). Cadmium induced the accumulation of poly(A)RNAs homologous to clones 53 (Fig. 2A), 75 (Fig. 2B) and 54 (Fig. 2C) to substantial levels in a concentration-dependent manner (lanes 5, 6 and 7); in the case of clones 53 and 75, somewhat lower levels of hybridizing RNA accumulated than in hs tissue (lane 2). The other metals did not detectably affect the concentration of those sequences (lanes 3,

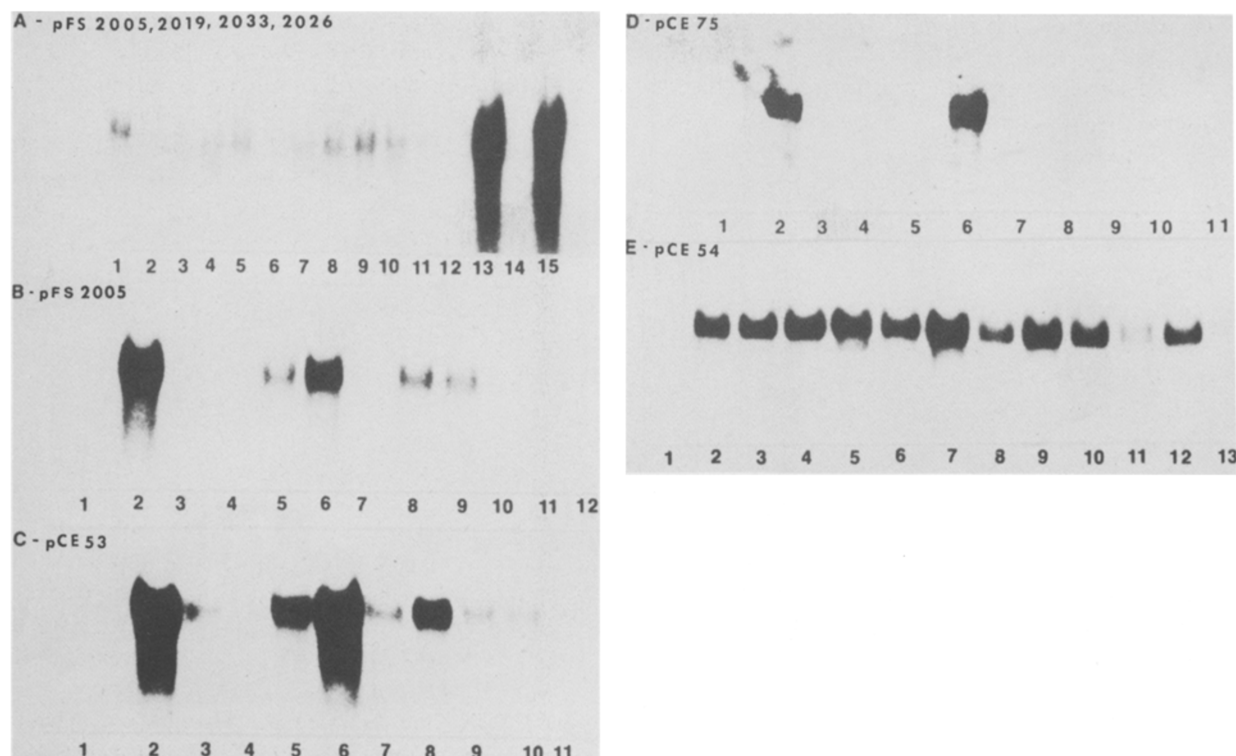

Fig. 1. Comparison of heat shock and other stresses on induction of hs mRNAs by northern blot analysis. One μg samples of poly(A)RNA isolated from intact soybean seedlings after treatment as noted were electrophoresed on 2% agarose/6% formaldehyde gels, blotted to nitrocellulose and hybridized to [^{32}P]-labelled plasmids containing cloned cDNAs: A) pFS 2005, pFS 2019, pFS 2033, pFS 2026; B) pFS 2005; C) pCE 53; D) pCE 75; E) pCE 54. The treatments:

A) 1 mM DNP, lane 1; 250 mM KCl, lane 2; 0.5 mM ABA, lane 3; 510 μg/ml 2,4-D, lane 4; N_2 gas treatment, lane 5; 1 °C cold shock, lane 6; −2 bars PEG, lane 7; −5 bars PEG, lane 8; −8 bars PEG, lane 9; −11 bars PEG, lane 10; −14 bars PEG, lane 11; control 28 °C, lanes 12 and 14; heat shock 40 °C, lanes 13 and 15;

B) and E) control 28° C, lane 1; heat shock 40° C, lane 2; 125 mM KCl, lane 3; 0.75 mM ABA, lane 4; 1 mM DNP, lane 5; 100 μM sodium arsenite, lane 6; 510 μg/ml 2,4-D, lane 7; 1 mM canavanine, lane 8; 1 mM p-fluorophenylalanine, lane 9; 0.1 mM $CoCl_2$, lane 10; N_2 gas, lane 11; −5 bars PEG, lane 12; and additionally Fig. E control 28 °C, lane 13;

C) and D) control 28 °C, lanes 1 and 11; treatments in lanes 2 to 8 are identical with ones described for B and E, N_2 treatment, lane 9; −5 bars PEG, lane 10.

Unless otherwise noted, treatments were for 2 h.

4, 8 and 9). With the possible exception of zinc, all of the metals enhanced slightly the level of poly(A)-RNA hybridizing to clone 54. Cadmium induced clone 54 RNA to levels similar to hs and additionally caused the accumulation of large amounts of a higher molecular weight RNA; this result has been obtained in repeated experiments (e.g. Fig. 3C) under a number of conditions. It is also apparent from Fig. 3A–F that cadmium induced the accumulation of poly(A)RNAs homologous to all of the cloned cDNAs used in these studies, although only very low levels homologous to clones pFS 2019 (Fig. 3D) and especially to pFS 2033 (Fig. 3F) were detected. A general summary of the influence of a number of other potential stress agents on hs mRNA levels based on northern blot analyses (data not shown) is presented in Table 1.

Further analysis of poly(A)RNAs homologous to clone 54

Clone 54 was used in northern blot hybridization analyses to assess the influence of a number of stress agents on the level of homologous poly(A)RNA

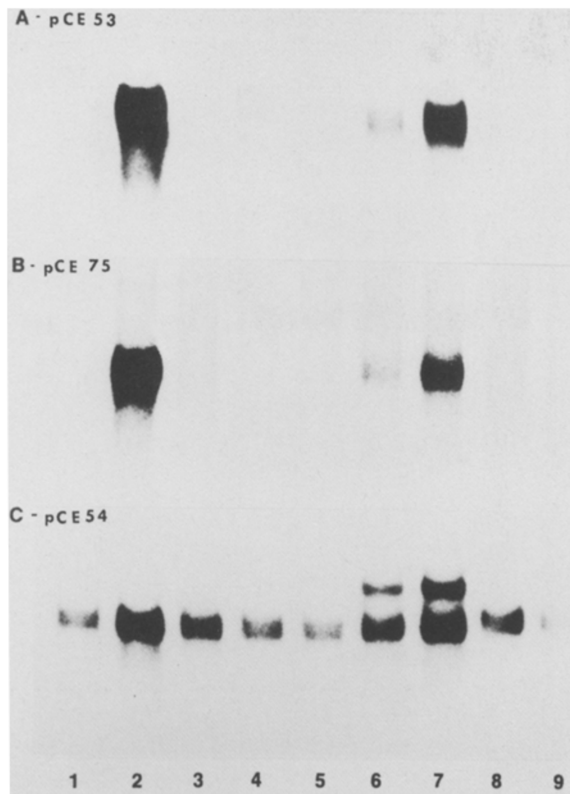

Fig. 2. Heavy metal induction of heat shock specific RNA as analyzed by northern blot hybridization. Poly(A)RNA was isolated from soybean seedlings incubated with various heavy metals for 2 h, electrophoresed under denaturing conditions, blotted to nitrocellulose, hybridized to [^{32}P]-labelled pCE 53 (A), pCE 75 (B) and pCE 54 (C). Metal salts (Cl^-) used were: aluminum 100 μM, lane 3; aluminum 250 μM, lane 4; cadmium 10 μM, lane 5; cadmium 100 μM, lane 6; cadmium 250 μM, lane 7; nickle 250 μM, lane 8; zinc 250 μM, lane 9. As controls 28 °C RNA (lane 1) and hs RNA from seedlings incubated for 2 h at 40 °C (lane 2) were used.

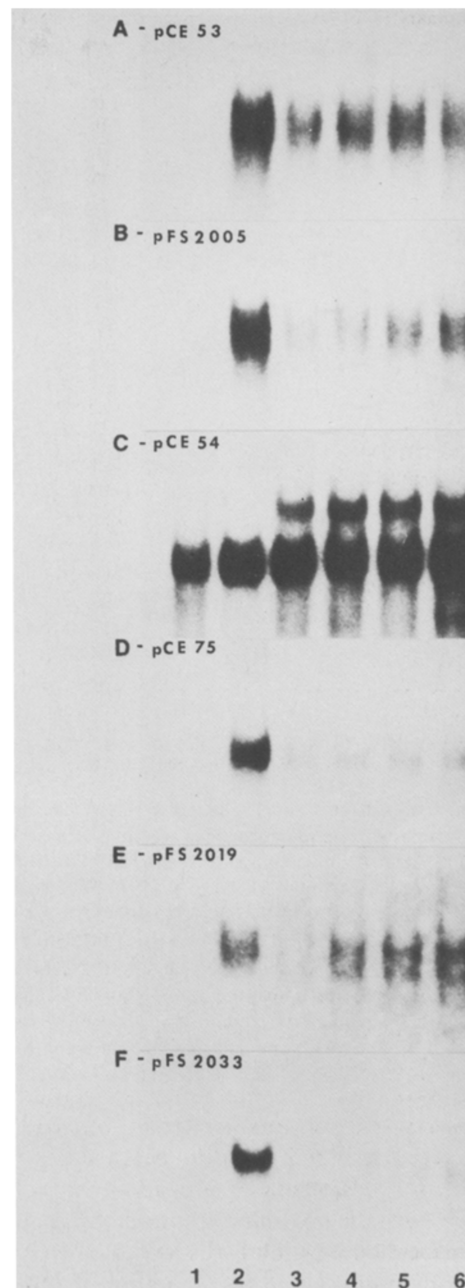

Fig. 3. Northern blot analysis of poly(A)RNA from soybean seedlings treated with cadmium. One μg poly(A)RNA samples electrophoresed on 2% agarose gel in denaturing conditions were blotted to nitrocellulose filters and hybridized to nick translated cDNA clones pCE 53 (A), pFS 2005 (B), pCE 54 (C), pCE 75 (D), pFS 2019 (E), pFS 2023 (F). Two h treatments were as follows: control 28 °C, lane 1; heat shock 40 °C, lane 2; 50 μM $CdCl_2$, lane 3; 100 μM $CdCl_2$, lane 4; 250 μM $CdCl_2$, lane 5; 500 μM $CdCl_2$, lane 6.

(Fig. 4). Lane 1 represents hybridization to 28 °C or control poly(A)RNA and lane 2 to 40 °C hs poly(A)RNA as standards of comparison in Fig. 4A–E. Heat shock results in the accumulation of a high level of hybridizing RNA above the 28 °C control level; the control level varies considerably from experiment to experiment, resulting in about a 3- to 10-fold increase in different experiments in response to hs. Anaerobiosis (Fig. 4A), water stress (Fig. 4B), dinitrophenol (Fig. 4C), salt stress (Fig. 4D), high inhibitory concentrations of 2,4-D (Fig. 4E) and abscisic acid (Fig. 4F and G) all cause

Table 1. Summary of the 'stress' induced accumulation of poly(A)RNAs homologous to hs-cDNA clones of soybean.

Treatment	pFS 2005	pFS 2019	pFS 2033	pCE 75	pCE 53	pCE 54
Control 28 °C 2 h	–	–	–	–	–	1+
Heat shock 40 °C 2 h	10+	8+	5+	5+	10+	5+
Arsenite 50, 100 μM	6+	6+	2+	4+	7+	5+
$CdCl_2$ 500 μM	4+	3+	1+	2+	5+	6+
2,4-D 510 μg/ml	1+	1+	NA	1+	1+	5+
C_2H_4 10 ppm	–	–	NA	–	–	–
GA_2 1 mM	–	–	NA	–	–	2+
Kinetin 1 mM	–	–	NA	–	–	2+
ABA 0.75 mM	–	–	NA	–	–	5+
PEG –5 bars, –8 bars, 2 h	–	–	NA	–	–	4+
KCl 125 mM 2 h	–	–	NA	–	–	4+
DNP 1 mM 2 h	1+	1+	NA	1+	T	5+
Anaerobiosis (N_2)	T	T	NA	NA	NA	2+
$CoCl_2$ 10, 100, 250 μM	–	–	NA	–	–	1+
NaF 10, 100, 1000 μM	–	1+	NA	–	T	4+
NaCN 10, 100, 1000 μM	–	–	NA	–	T	–
Canavanine 100 μM	T	T	NA	T	1+	5+
pFΦALA	–	–	NA	–	–	3+
Azetidine-2-COOH 500 μM	3+	2+	NA	NA	NA	4+
$AgCl_2$ 250 μM	T	T	NA	T	1+	1+
$NiCl_2$ 250 μM	–	–	NA	–	–	1+
$CuCl_2$ 250 μM	2+	1+	NA	1+	1+	1+

– = not detected.
NA = not analyzed.
\+ = estimated relative level from northern hybridization analyses.
T = trace.

significant increases in the level of poly(A)RNA homologous to clone 54. It is not known whether the results obtained with PEG and KCl relate only to effects on water relations or whether KCl relates also to a more specific salt effect. While the level of the RNA which accumulates in response to the various stresses varies with time of treatment and severity of the stress, all of these stress conditions cause a several-fold increase of hybridizing RNA above control levels, resulting in levels similar to that induced by hs. The data presented in Fig. 4F and G provide a comparative analysis of 'dot' hybridization with the 'northern' hybridization for the response to ABA. The two methods provide similar results of the influence of ABA on the level of poly(A)RNA homologous to clone 54. While a more precise estimate of the (semi)quantitative effect of ABA on the concentration of this sequence may be possible with the 'dot' analysis, a somewhat higher level of confidence (to us at least) in the specificity of hybridization is offered by the 'northern hybridization'. While 'dot' hybridizations were made on other 'stress' RNAs, only 'northern' hybridization data have been presented for the other stresses and other cloned cDNAs.

Since a wide range of stress agents led to an enhanced accumulation of poly(A)RNA homologous to clone 54, this clone was used to hybrid select that RNA to determine if similar proteins were translated from those poly(A)RNAs. The hybridized RNA was then subjected to *in vitro* translation in the wheat germ system. Figure 5 shows the 2D gel pattern of the translation products of those hybrid selected poly(A)RNAs isolated from tissue stressed by a number of different agents. The two circled spots represent endogenous translation products of the wheat germ system used in these experiments. The translation products of the hybrid selected RNAs migrated as four 27 Kd protein spots (arrows), one to the extreme left of the gels and the other three migrating near the middle of the gels (pH gradient from about 4 on left to 7.5 on right). The translation products appear to be electrophoretically identical for the RNAs hybrid selected

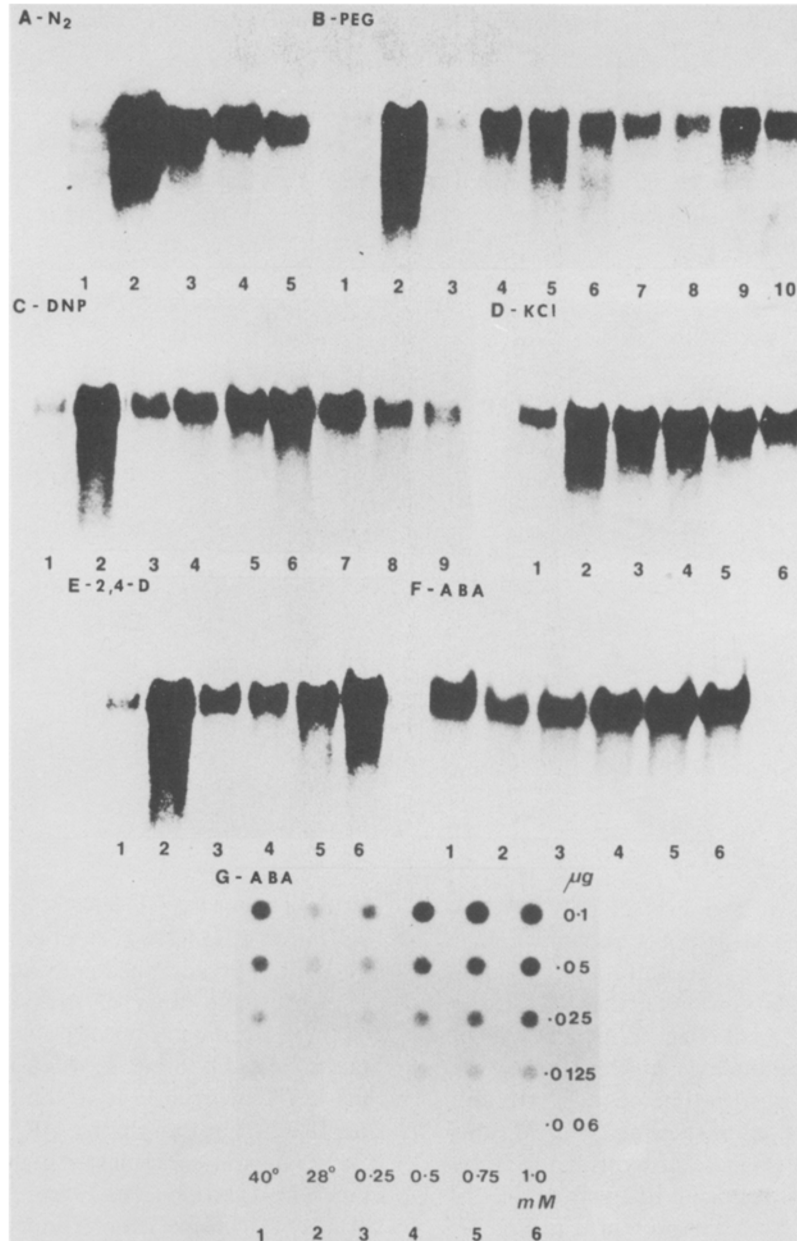

Fig. 4. Northern and dot blot hybridization analysis of poly(A)$^+$RNA from soybean seedlings stressed by various environmental conditions. Poly(A)$^+$RNA samples (1 μg) were electrophoresed on 2% agarose/6% formaldehyde gels, blotted to nitrocellulose filters and hybridized to [^{32}P]-labelled plasmid pCE 54. Control (28 °C) and heat shock (40 °C) poly(A)$^+$RNA are shown in lanes 1 and 2, respectively, on each northern blot unless otherwise stated.

Time course of anaerobiosis (A): 2 h, lane 3; 7 h, lane 4; 21 h, lane 5.

Low water potential (B): 2 h incubation at -2 bars PEG, lane 3; -5 bars PEG, lane 4; -8 bars PEG, lane 5; seedlings were incubated at -5 bars PEG for 3 h, lane 6; 7 h, lane 7; 21 h, lane 8 or at -8 bars PEG for 3 h, lane 9; and 7 h, lane 10.

Dinitrophenol (C): seedlings were incubated for 15 min, lane 3; 30 min, lane 4; 1 h, lane 5; 2 h, lane 6, in medium containing 1 mM DNP or increasing concentrations of DNP i.e. 2 mM, lane 7; 3 mM, lane 8; 4 mM, lane 9 for 2 h.

KCl treatment (D): 125 mM, lane 3; 250 mM, lane 4; 375 mM, lane 5; 500 mM, lane 6 for 2 h.

Auxin (2,4-D) treatment (E): intact seedlings were incubated in medium containing 510 μg/ml of 2,4-D for 15 min, lane 3; 30 min, lane 4; 1 h, lane 5; 2 h, lane 6.

ABA treatment (F, G): heat shock, lane 1; control, lane 2; 0.25 mM, lane 3; 0.5 mM, lane 4; 0.75 mM, lane 5; 1 mM (lane 6).

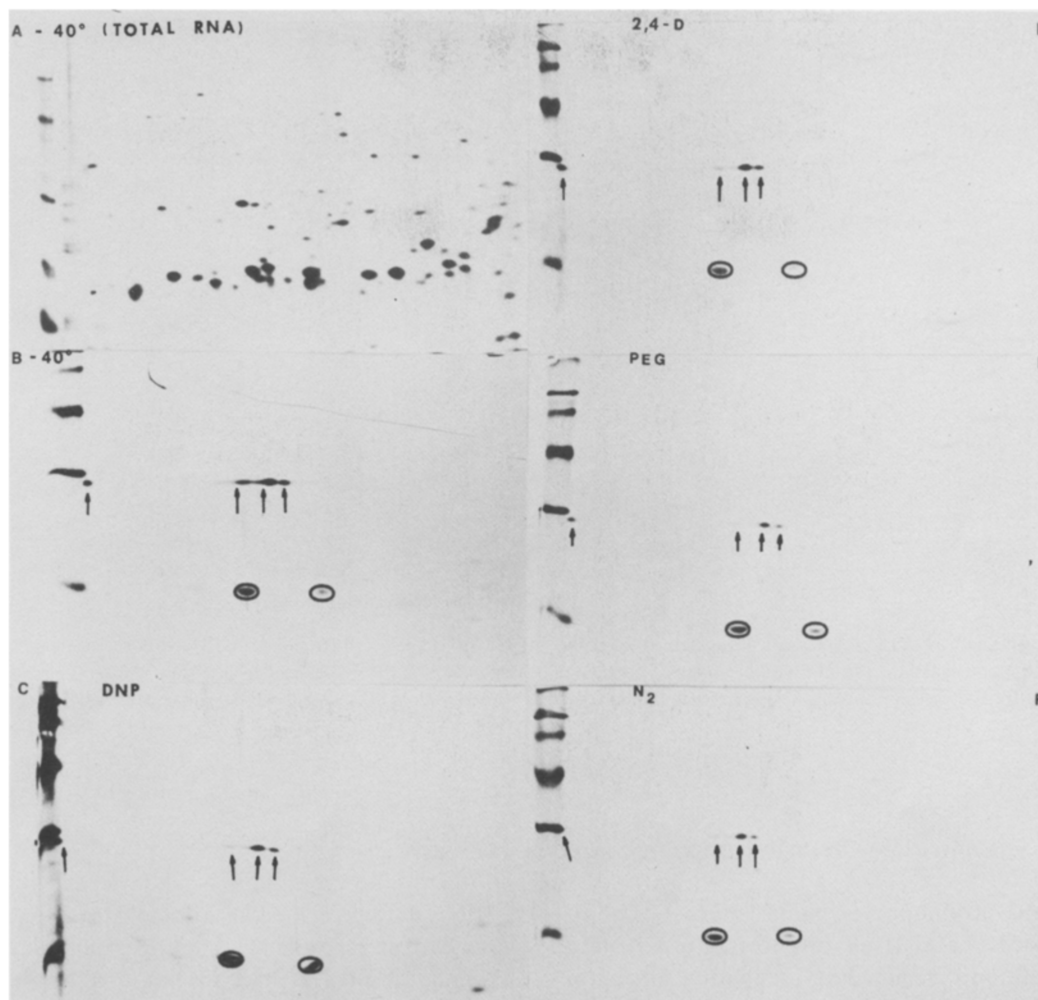

Fig. 5. 2D gel analysis of *in vitro* translation products of hybrid-selected RNA. Translation products from total poly(A)RNA from 40 °C hs tissue are shown in Fig. 5A. RNA was hybrid selected by recombinant plasmid pCE 54 from total poly(A)RNA obtained from seedlings stressed by: B, 40 °C heat shock; C, 1 mM DNP; D, 510 μg/ml 2,4-D; E, -8 bars of PEG; F, anaerobiosis.
The hybrid released RNA was translated *in vitro* in the S-30 wheat germ system. Proteins were separated on O'Farrell 2D gels. Markers of molecular weight 92 500, 69 000, 46 000, 30 000, 12 300 daltons are shown on the left side of each gel. Circled spots identify major endogenous S-30 wheat germ translation products. Arrows indicate translation products of hybrid selected poly(A)RNA.

from tissue treated with hs at 40 °C (Fig. 5B), DNP (Fig. 5C), 2,4-D (Fig. 5D), -8 bars PEG (Fig. 5E), and anaerobic conditions (Fig. 5F). These same translation products were seen in the 2D gel analysis of total poly(A)RNA (Fig. 5A). A similar set of 27 Kd translation products was obtained when the poly(A)RNAs were hybrid selected from cadmium-treated tissue (Fig. 6). Additionally, two major low molecular weight translation products (double arrows) were present on 2D gels from RNAs hybrid selected from cadmium-treated tissues that were not present in the other stress treatments (Fig. 5). (It should also be noted that the circled endogenous translation products of Fig. 5 are not present in Fig. 6 because of an appropriate micrococcal nuclease treatment to degrade endogenous wheat germ mRNAs not used in the earlier experiments (Fig. 5A–F)). These additional translation products may relate to the additional poly(A)RNA band noted in northern blots of RNA from cadmium-treated tissue, although we have no direct evidence for this.

Fig. 6. 2D gel analysis of *in vitro* translation products of poly(A)RNA hybrid selected from total poly(A)RNA from $CdCl_2$-treated tissue. RNA was selected by recombinant plasmid pCE 54 and translated *in vitro* in the S-30 wheat germ system. Proteins were separated by 2-D O'Farrell gel electrophoresis. Molecular weight markers of 92 500, 69 000, 46 000, 30 000, 12 300 dalton are shown on the left side of the gel.

Possible role of ethylene in the hs(stress) response

Ethylene production in plants increases significantly above basal levels following stress from a variety of sources including incubation in phytotoxic chemicals, especially cadmium (1, 17, 22), supraoptimal auxin concentrations (11, 23), or an osmoticum (49). In general, ethylene production increases at temperatures up to 35 °C, but is completely prevented at higher temperatures (52, 62). Although we did not measure the rate of ethylene production, our hs-treated seedlings did show some morphological characteristics of ethylene-treated plants. Therefore, influence of exogenous ethylene on the induction of poly(A)RNAs homologous to hs-specific clones and on the induction of those RNAs by hs was assessed (Fig. 7). The data presented in lanes 1–4 show that ethylene did not affect the level of poly(A)RNAs homologous to clones 2005, 53, 75 and 54 (Fig. 7). Further, the data presented in lanes 5–7 (Fig. 7) demonstrate that ethylene or an inhibitor of endogenous ethylene production, AVG (63), had no effect on the hs-induced accumulation of these RNAs.

Discussion

The data presented here demonstrate that a wide range of stress agents, including a number of environmental stresses often encountered by plants, induce to varying extents the accumulation of poly(A)RNAs which are also induced rapidly and to very high levels by a heat shock treatment of soybean seedlings (54). With only one or two exceptions (e.g. arsenite and possibly cadmium), the levels of poly(A)RNA which accumulate during these short term 'stress' treatments are relatively low compared to the level found in hs tissue for the group of hs cDNA clones used in these experiments (clones 2005, 2019, 2033, 53 and 75); in many cases detectable levels were not even induced. Thus, such common environmental stresses such as water stress, salt stress, anaerobiosis and several heavy metals do not mimic hs in inducing similar levels of the same mRNAs and hs proteins. Arsenite and cadmium do induce significant levels of the other hs mRNAs in contrast to most of the other 'stresses' tested. The exception to these observations on the variable and low level induction of poly(A)RNAs

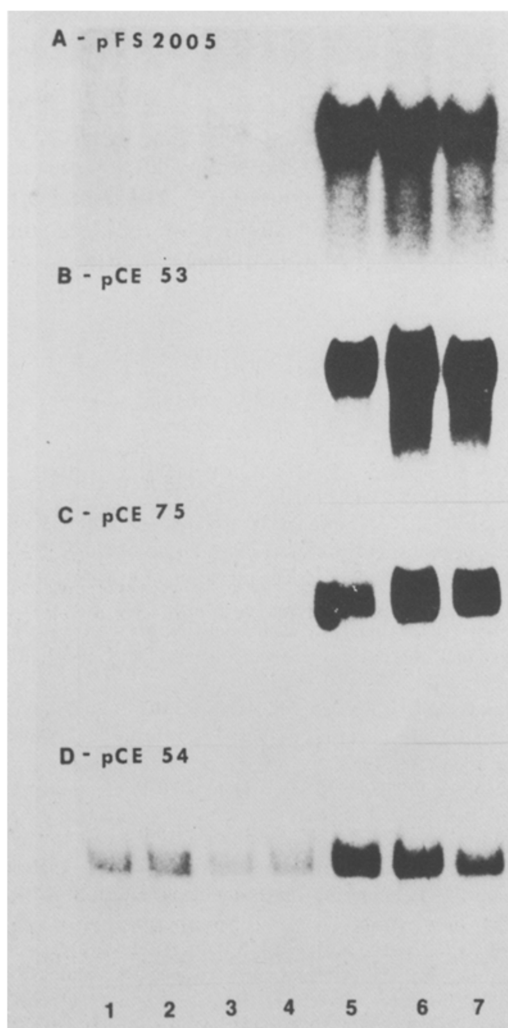

Fig. 7. Relationship of ethylene to the heat shock response of soybean seedlings as investigated by northern blot hybridization analyses. Soybean seedlings were incubated in buffer A with ethylene (10 ppm) and/or air mixture bubbling through the solution. Poly(A)RNA was electrophoresed on 2% agarose/6% formaldehyde gels, and after blotting to nitrocellulose filters hybridized to pFS 2005 (A), pCE 53 (B), pCE 75 (C), pCE 54 (D). Treatments were as follows: 28 °C with an air flow through the incubation solution, lane 1; the same as lane 1 plus 10 μM AVG in the incubation medium, lane 2; ethylene and air flow through the medium, lane 3; ethylene and air flow through the standard incubation mix containing 10 μM AVG, lane 4; 40 °C with air flow, lane 5; 40 °C with air flow through medium containing 10 μM AVG, lane 6; the same as lane 6 except that AVG concentration was 100 μM, lane 7.

homologous to hs cDNA clones relates to the results obtained when clone 54 was used as the radioactive hybridization probe in northern blot hybridization analyses. In contrast to the other cloned cDNAs noted above, control (presumably unstressed) tissues contained significant and somewhat variable levels of poly(A)RNAs homologous to clone 54. Heat shock and most of the other stresses analyzed here simply caused a 3- to 10-fold increase in the concentration of the poly(A)mRNAs hybridizing to clone 54. Of the many agents tested (see Table 1) only a small number did not significantly affect the relative level of RNAs hybridizing to clone 54. Additionally, cadmium induced the accumulation of a second higher molecular weight poly(A)RNA which hybridized to clone 54 along with the increased level of the normal band of poly(A)RNA (that which translates into the group of 27 Kd proteins). Thus clone 54 is unique among the hs clones used in our work. First, RNAs homologous to this cloned sequence are present in all control (non-stressed) soybean tissue analyzed to date and second, most 'stress' agents induce the accumulation of these RNAs to levels near the hs-induced level. The physiological significance of these observations is not known. It seems possible, however, that the 27 Kd proteins, or their gene family, are somehow involved in sensing stress or in modulating the cellular response to stress. These appear to be soluble proteins and not a part of the family of hs proteins which relocalize within the cell in response to hs and recovery from hs (29, 30). Their presence in 'unstressed' tissues, however, makes it possible that these proteins could be associated with the early regulatory events induced by stress.

The working model when these studies were initiated was that a regulatory mechanism(s) might become operative in response to a wide range of environmental (physical) stresses similar to the one(s) which lead to the dramatic shift in patterns of protein synthesis induced by hs (28) and anaerobiosis (51) in plants and known to function in *Drosophila* (3). While these responses share many common features as outlined in the introduction, the actual 2D gel patterns of anaerobic and hs proteins are quite different as likely are their functions (e.g. 28, 51). The northern analyses presented here using the cloned hs cDNAs show that anaerobiosis does not induce significant levels of hs mRNAs, excepting the results with clone 54. However, a low rate of

accumulation of these 'hs mRNAs' over an extended period of partial anaerobiosis might have some physiological significance. The data do indicate that the regulatory system(s) which causes the transcription response to hs must in some way 'sense' the imposition of many of the 'stresses' used in these studies since detectable levels (above controls) of most of these mRNAs do accumulate. Yet only arsenite and cadmium seem to elicit a sufficiently large transcriptional response of the 'hs genes' to have short term physiological significance; other work (Lin, Roberts & Key, in preparation) shows that the response to arsenite does have physiological significance in that arsenite treatment leads to the development of thermal protection to otherwise non-permissive hs temperatures (e.g. 45 °C) similarly to a 40° C hs treatment (29, 30). The cadmium response may be similar to that of arsenite but has not yet been studied beyond the data presented here.

As noted in the introduction, we have used hs cDNA clones to assess whether a wide range of stress agents mimic hs in preference to amino acid incorporation/ gel analysis of proteins, since amino acid uptake is so dramatically reduced by many of the stresses, thus the northern hybridization analyses allow for a much more quantitative analysis of the relationships. We do know, however, that clone pCE 54 mRNAs are expressed *in vivo* in control and hs tissues (e.g. 28, 30). The limited *in vivo* analyses made to date on expression *in vivo* of clone 54 proteins do show significant expression of these proteins along with high levels of amino acid incorporation into protein in arsenite- and 2,4-D-treated tissues (unpublished data); the 27 kD band is a dominant band after long exposures of the gels of *in vivo* proteins from water stressed, anaerobic treated, and DNP-treated tissues where amino acid incorporation is exceedingly low, bringing into question the over-all validity of the analyses. The accumulated pCE 54 mRNAs from all of those tissues are translatable *in vitro*, and likely so *in vivo* as well. Additional studies of *in vivo* protein synthesis will be made with a number of these stresses in the near future.

An often raised question about the hs response in plants relates to the possible role of ethylene in the response. The data presented here indicate that ethylene plays no obvious role in the hs response. It may be, however, that enhanced ethylene production is a consequence of hs since hs seedlings show some characteristics of ethylene-treated soybean seedlings.

Acknowledgements

This research was supported by DOE contract 10-21-RR167-090. We thank Dr C. Y. Lin and Michael Mansfield for discussions of the data and assistance in 2D gel analysis of the translation products.

References

1. Abeles AL, Abeles FB: Biochemical pathway of stress-induced ethylene. Plant Physiol 50:496, 1972.
2. Altschuler M, Mascarenhas JP: Heat shock proteins and effects of heat shock in plants. Plant Mol Biol 1:103, 1982.
3. Ashburner N, Bonner JF: The induction of gene activity in Drosophila by heat shock. Cell 17:241, 1979.
4. Barnett T, Altschuler M, McDaniel CN, Mascarenhas JP: Heat shock induced proteins in plant cells. Dev Genet 1:331, 1980.
5. Baszczynski CL, Walden DB, Atkinson BG: Regulation of gene expression in corn (Zea mays L.) by heat shock. Can J Biochem 60:569, 1982.
6. Baulcombe DB, Key JL: Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyls. J Biol Chem 255:8907, 1980.
7. Bazaral M, Helinski DK: Circular forms of colicinogenic factors E1, E2 and E3 from Escherichia coli. J Mol Biol 36:185, 1968.
8. Bewley FD, Larsen KM: Cessation of protein synthesis in water-stressed pea roots and maize mesocotyls without loss of poly-ribosomes. Effects of lethal and non-lethal water stress. J Exp Bot 31:1245, 1980.
9. Bishop JO, Rosbach M, Evans D: Polynucleotide sequence in eucaryotic DNA and RNA that form ribonuclease resistant complexes with polyuridylic acid. J Mol Biol 85:75, 1974.
10. Botha FC, Botha PT: The effect of water stress on the nitrogen metabolism of two maize lines. Effects on the rate of protein synthesis and chlorophyll content. J Pflanzenphysiol Bol 94:179, 1979.
11. Burg SP, Burg EA: The interaction between auxin and ethylene and its role in plant growth. Proc Natl Acad Sci 55: 262, 1966.
12. Cooper P, Ho TD: Heat shock proteins in maize. Plant Physiol 71:215, 1983.
13. Ellgaard EG: Similarities in chromosomal puffing induced by temperature shocks and dinitrophenol in Drosophila. Chromosoma 37:417, 1972.
14. Ferl RJ, Dlouhy SR, Schwartz D: Analysis of maize alcohol dehydrogenase by native-SDS two dimensional electrophoresis and autoradiography. Mol Gen Genet 169:7, 1979.

15. Francis D, Lin L: Heat shock response in a cellular slime mold, Polysphondylium palladium. Dev Biol 79:238, 1980.
16. Freeling M: Simultaneous induction by anaerobiosis and 2,4-D of multiple enzymes specified by two unlinked genes: differential Adhl-Adh2 expression in maize. Molec Gen Genet 127:215, 1973.
17. Fuhrer J: Ethylene biosynthesis and cadmium toxicity in leaf tissue of beans (Phaseolus vulgaris, L). Plant Physiol 70:162, 1982.
18. Gerlach WL, Pryor AF, Dennis ES, Ferl RF, Sachs MM, Peacock WF: cDNA cloning and induction of alcohol dehydrogenase gene (Adhl) of maize. Proc Natl Acad Sci USA 79:2981, 1982.
19. Glover CVC, Varna KF, Guttman SD, Gorovsky MA: Heat shock and deciliation induce phosphorylation of histone H1 in T. pyriformis. Cell 23:73, 1981.
20. Guttman SD, Glover CVC, Allis CD, Gorovsky MA: Heat shock deciliation and release from anoxia induce the synthesis of the same set of polypeptides in starved T. pyriformis. Cell 22:299, 1980.
21. Hershfield V, Boyer NW, Yanofsky C, Lovett MA, Helinski DR: Plasmid ColEl as molecular vehicle for cloning and amplification of DNA. Proc Natl Acad Sci USA 71:3455, 1974.
22. Hogsett WE, Raba RM, Tingey DT: Biosynthesis of stress ethylene in soybean seedlings: similarities to endogenous ethylene biosynthesis. Plant Physiol 53:307, 1981.
23. Holm RE, Abeles FB: The role of ethylene in 2,4-D-induced growth inhibition. Planta 78:293, 1968.
24. Hsiao TC: Rapid changes in levels of polyribosomes in Zea mays in response to water stress. Plant Physiol 46:281, 1970.
25. Jackson M, Ingle J: The interpretation of studies on rapidly labeled ribonucleic acid in higher plants. Plant Physiol 51:412, 1973.
26. Johnston D, Opperman H, Jackson J, Levinson W: Induction of proteins and mRNA in chick cells by arsenite. J Biol Chem 255:2975, 1980.
27. Kelley PM, Schlesinger MJ: The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell 15:1277, 1978.
28. Key JL, Lin CY, Chen YM: Heat shock proteins of higher plants. Proc Natl Acad Sci USA 78:3526, 1981.
29. Key JL, Lin CY, Ceglarz E, Schöffl F: Heat shock from bacteria to man. In: Schlesinger MJ, Ashburner M, Tissières A (eds) The heat shock response in plants: physiological considerations. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, p 329.
30. Key JL, Lin CY, Ceglarz E, Schöffl F: The heat shock response in soybean seedlings. In: Dure L (ed) NATO Advanced Studies Workshop on Genome Organization and Expression in Plants. Plenum Press. In press, 1983.
31. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacterio-phase T4. Nature 227:680, 1970.
32. Levinson W, Opperman H, Jackson J: Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. Biochim Biophys Acta 606:170, 1980.
33. Li GC, Werb L: Correlation between synthesis of heat shock proteins and development of thermotolerance in chinese hamster fibroblasts. Proc Natl Acad Sci 79:3218, 1982.
34. Lin CY, Key JL: Dissociation and reassembly of polyribosomes in relation to protein synthesis in the soybean root. J Mol Biol 26:237, 1967.
35. Loomis WF, Wheeler S: Heat shock response in Dictyostelium. Dev Biol 79:399, 1980.
36. Loomis WF, Wheeler SA: Chromatin-associated heat shock proteins of Dictyostelium. Dev Biol 90:412, 1982.
37. McAlister L, Finkelstein D: Heat shock proteins and thermal resistance in yeast. Biochem Biophys Res Commun 93:819, 1980.
38. McGrogan M, Spector DJ, Goldenberg CJ, Halbert D, Raskas HF: Purification of specific adenovirus 2 RNAs by preparative hybridization and selective thermal elution. Nucleic Acids Res 6:593, 1979.
39. Michel BE: Carbowax 6000 compared with mannitol as a suppressant of cucumber hypocotyl elongation. Plant Physiol 45:507, 1970.
40. Mitchell H, Moller G, Petersen N, Lipps-Sarmiento L: Specific protection from phenocopy induction by heat shock. Dev Genet 1:181, 1979.
41. Mocquot B, Prat CH, Mouches C, Pradet A: Effect of anoxia on energy charge and protein synthesis in rice embryo. Plant Physiol 68:636, 1981.
42. Molecular cloning. A laboratory manual. In: Maniatis T, Fritsch ER, Sambrook J (eds) Spun column procedure. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, p 466.
43. Moriella CA, Boyer JS, Hageman RH: Nitrate reductase activity and polyribosomal content of corn (Zea mays L.) having low leaf water potentials. Plant Physiol 51:817, 1973.
44. Neidhardt FC, Van Bogelen RA: Positive regulatory gene for temperature-controlled proteins in Escherichia coli. Biochem Biophys Res Commun 100:894, 1981.
45. O'Farrel PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007, 1975.
46. Raskas HF, Green M: In: Koprowski H, Maramorosch K (eds) Methods in virology. Academic Press, Inc., New York, NY, 1971, p 252.
47. Rave N, Crkvenjakov R, Boedtker H: Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucleic Acids Res 6:3559, 1979.
48. Rigby PWJ, Dieckmann M, Rhodes C, Beng P: Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237, 1977.
49. Riov J, Yang SF: Stimulation of ethylene production in citrus leaf discs by mannitol. Plant Physiol 70:142, 1982.
50. Ritosa F: A new puffing pattern induced by heat shock and DNP in Drosophila. Experientia 18:571, 1962.
51. Sachs MM, Freeling M, Okimoto, R: The anaerobic proteins of maize. Cell 20:761, 1980.
52. Saltveit ME, Dilley DR: Rapidly induced wound ethylene from excised segments of Pisum sativum L., cv. Alaska. II. Oxygen and temperature dependency. Plant Physiol 61:675, 1978.

53. Scharf K-D, Nover L: Heat shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell 30:427, 1982.
54. Schöffl F, Key JL: An analysis of mRNAs for a group of heat shock proteins of soybean using cloned cDNAs. J Molec Appl Genet 1:301, 1982.
55. Scott MP, Pardue ML: Translational control in lysates of Drosophila melanogaster cells. Proc Natl Acad Sci USA 78:3353, 1981.
56. Silflow CD, Hammett JR, Key JL: Sequence complexity of polyadenylated ribonucleic acid from soybean suspension culture cells. Biochemistry 13:2725, 1979.
57. Storti RV, Scott MP, Rick A, Pardue ML: Translational control of protein synthesis in response to heat shock in D. melanogaster cells. Cell 22:825, 1980.
58. Swanstrom R, Shank PK: X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. Anal Biochem 86:184, 1978.
59. Thomas PS: Hybridization of denatured RNA and small DNA fragments to nitrocellulose. Proc Natl Acad Sci USA 77:5201, 1980.
60. Wang Ch, Gomer RH, Lazarides E: Heat shock proteins are methylated in avian and mammalian cells. Proc Natl Acad Sci USA 78:3531, 1981.
61. Yamamori T, Yura T: Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in Escherichia coli K-12. Proc Natl Acad Sci 79:860, 1982.
62. Yu Y-B, Adams DO, Yang SF: Inhibition of ethylene production by 2,4-dinitrophenol and high temperature. Plant Physiol 66:286, 1980.
63. Yu Y-B, Yang SF: Auxin induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Physiol 64:1074, 1979.

Received 25 July 1983; in revised form and accepted 18 October 1983.